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THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket: BARKAN=2

In re Application of:

Dalit BARKAN et al.

Appln. No.: 09/403,897

Filed: February 22, 2000

For: LEPTIN AS AN INHIBITOR OF
TUMOR CELL PROLIFERATION

Conf. No.: 7830

Art Unit: 1642

Examiner: K. Canella

Washington, D.C.

May 30, 2003

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BRIEF ON APPEAL

Honorable Commissioner for Patents
U.S. Patent and Trademark Office
2011 South Clark Place
Customer Window, Mail Stop Appeal Brief-Patents
Crystal Plaza Two, Lobby, Room 1B03
Arlington, VA 22202

Sir:

Submitted herewith is applicant's Brief on Appeal in triplicate.

The present appeal is taken from the action of the examiner in finally rejecting claims 2-8, 28, 30-34 and 36-39. The full text of the claims 2-8, 28, 30-34 and 36-39 appears in Appendix A attached hereto. The full text of allowed claims 9, 29, and 35 appears in Appendix B attached hereto.

REAL PARTY IN INTEREST

The present application is owned by Yeda Research and

Development Co. Ltd., which is the research and development arm

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of the Weizmann Institute of Science in Rehovot, Israel. The exclusive licensee of the present invention is Inter-Lab Limited, an Israeli company of Ness-Ziona, Israel. Inter-Lab Limited is a subsidiary of InterPharm Laboratories Limited, an Israeli company of Ness-Ziona, Israel, which is a subsidiary of Serono B.V., whose parent company, Serono S.A., is a holding company under which there are many subsidiaries worldwide.

RELATED APPEALS AND INTERFERENCES

Appellant is aware of no other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the present appeal.

STATUS OF CLAIMS

Claims 2-9 and 28-39 presently appear in this case. Claims 2-8, 28, 30-34 and 36-39 are under final rejection. Claims 9, 29 and 35 have been allowed. Claims 21 and 10-27 have been cancelled without prejudice.

STATUS OF AMENDMENTS

The most recent rejection in this case was the final rejection of July 30, 2002. No amendments to the claims have been filed subsequent to that date.

SUMMARY OF THE INVENTION

Leptin is a cytokine that is derived from adipocytes, which is known to regulate body weight (page 1, line 10¹). Insulin has been reported to reduce significantly the basal and insulin-induced tyrosine-phosphorylation of the insulin receptor substrate-1 (IRS-1) (page 1, lines 27-29). The present invention is based on the concept of using leptin as an inhibitor of cell proliferation for the treatment of various malignancies, preferably for the inhibition of human breast carcinoma cell proliferation (page 2, lines 5-8). The proliferation of many types of tumor cells is increased in the presence of various growth factors, such as insulin and Insulin-like Growth Factor-I (IGF-I). Leptin inhibits the pathway that mediates the growth stimulatory effect of insulin and IGF-I on cells (page 2, lines 9-12).

Besides leptin, which is a known and well-defined protein (page 1, lines 10-13), muteins of leptin having at least 60% identity with the sequence of leptin may be used, as long as such mutein has the ability to block cell proliferation (page 8, line 19, to page 9, line 3). Alternatively, the mutein may be one having a sequence encoded by a nucleic acid which hybridizes to a nucleic acid that encodes leptin under

¹ All page and line citations in this section are to the present specification.

stringent conditions and has the ability to block cell proliferation (page 13, lines 8-23).

The method of the present invention may also use a fragment of a leptin mutein as defined above as long as that fragment has the ability to block cell proliferation (page 14, lines 21-25). Fusion proteins of the above-defined leptin mutein or fragment may also be used in the process of the present invention (page 13, lines 24-27).

Besides leptin and its muteins and fragments, either alone or in the form of a fusion protein, the method of the present invention can be accomplished using a leptin receptor agonist that has the ability to block cell proliferation (page 14, line 26, to page 15, line 19).

Salts and functional derivatives of any of the agents described above may also be used in the method of the present invention (page 13, line 28, to page 14, line 20).

In a preferred embodiment, the active agent is a mutein of leptin having at least 70% identity, more preferably at least 80%, and most preferably at least 90% identity with the sequence of a leptin, each of which having the ability to block cell proliferation (page 9, lines 1-3).

Claims directed to a method for treating tumors in mammals or for inhibiting tumor cell proliferation in mammals comprising administering to a mammal in need thereof an

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effective amount of an active agent which is leptin or a fusion protein comprising leptin have been allowed (see the final rejection of July 30, 2002, page 1, paragraph 5).

THE PRIOR ART

The final rejection of July 30, 2002, contains no rejections over the prior art. However, the enablement rejection refers to Bjorbaek et al, "Divergent Signaling Capacities of the Long and Short Isoforms of the Leptin Receptor", J. Biol. Chem., 272:32686-32695 (December 19, 1997). It should be noted that, as the effective filing date of the present application is April 29, 1997, this publication was not available to the art at the time the present invention was made.

The examiner cites this publication for the thesis that "in order for the leptin to exert it [sic] effect of inhibiting the phosphorylation of insulin receptor substrate-1, it must bind to the leptin receptor to activate JAK-2" (Final rejection of July 30, 2002, page 2).

The Bjorbaek publication, in the paragraph bridging pages 32689-32690 contains a section under the heading:

Both long and short forms of the leptin receptor can mediate tyrosine-phosphorylation of IRS-1

This section points out that, in addition to insulin, several other ligands and several interleukins can stimulate tyrosine-

phosphorylation of IRS-1 after occupancy of their own receptors, most likely via JAK kinases. Leptin is not listed among those other ligands. This section reports on tests which were conducted to evaluate the ability of leptin receptors to stimulate tyrosine-phosphorylation of IRS-1. The experiment involved co-transfection of human embryonal kidney (293) cells with the long form of the leptin receptor (OBR1) and IRS-1 expression vectors. Leptin failed to activate IRS-1 phosphorylation in those cells. In contrast, leptin strongly simulated tyrosine-phosphorylation of IRS-1 in cells co-transfected with OBR1, IRS-1, and either JAK-2 or JAK-1 cDNAs.

Thus, this reference relates to situations where leptin can stimulate tyrosine-phosphorylation of IRS-1. To the contrary, the present invention relates to the inhibition of the growth stimulatory effect of insulin and IGF-1 on tumor cells and is mediated by IRS-1 (page 4, lines 7-10 of the present specification).

THE REJECTIONS

Claims 2-8, 28, 30-34 and 36-39 have been rejected under 35 U.S.C. 112, first paragraph, the examiner stating:

[T]he specification, while being enabling for leptin and leptin-fusion proteins, does not reasonably provide enablement for leptin muteins, leptin receptor agonists, active fragments or fractions of anyone thereof, active analogs or derivatives of any thereof, and mixtures of any thereof as

inhibitors of tumor cell proliferation is maintained for reasons of record. It has been stated in the original rejection of Paper No. 10, mailed February 28, 2001, that in order for the leptin to exert its effect of inhibiting the phosphorylation of insulin receptor substrate-1, it must bind to the leptin receptor to activate JAK-2 (see: Bjorbaek et al, J. of Biological Chemistry, 1997). The instant specification provides only examples and guidance for the use of leptin as an inhibitor of the phosphorylation of insulin receptor substrate-1. Although having an intact leptin protein fused to another protein would have a reasonable expectation of binding the leptin receptor and activating the JAK-2 in the same manner as leptin, one of skill in the art would not know what changes in the leptin sequence could be tolerated by the leptin receptor with respect to the JAK-2 activation. Therefore, practice of this invention to the full scope of the claims would require undue experimentation to make and use substances other than leptin or leptin-fusion proteins. Further it is well known in the art that receptor antagonists need not share structural similarities. For instance, Maxadilan, a peptide derived from sand flies, is an agonist at the pituitary cyclase-activating peptide type I receptor, but bears no structural homology to PACAP. Thus it is not possible to predict the structural requirement necessary to both bind to the leptin receptor and activate JAK-2 in the same manner as leptin. Stating that the broadly claimed agonists and fragments have at least 60% identity to leptin is not a disclosure of how to alter the amino acid sequence of leptin in order to obtain muteins, fragments of agonists that would function as claimed. Muteins are defined on page 8 of the specification, active fragment are defined on page 14 and agonists on pages 14-15. However the specification relies only on general definitions, and does not teach one of skill

in the art how to alter the amino acid sequence of leptin in order to obtain mutein, active fragment or agonist that would function as claimed.

ISSUES

The following issue is presented in this appeal:

Does the disclosure as filed contain sufficient information regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and use the claimed invention?

GROUPING OF CLAIMS

Claims 28 and 2-8 stand or fall together. Each of claims 30, 31, 32, 33, 34, 36, 37, 38 and 39 must be individually considered for compliance with the enablement requirement with respect to the breadth of each of those claims.

A R G U M E N T

It Would Not Take Undue Experimentation to Practice the Full Scope of the Invention Claimed in Claim 39

The enablement requirement of 35 USC 112 is discussed at section 2164 et seq of the MPEP. MPEP §2164.01 states that any analysis of whether a particular claim is supported by the disclosure in an application requires a determination of whether that disclosure, when filed, contains sufficient information regarding the subject matter of the

claims as to enable one skilled in the pertinent art to make and use the claimed invention. The question is whether the experimentation needed to practice the invention is undue or unreasonable. If the invention can be practiced without undue or unreasonable experimentation, the enablement requirement is considered to be met. The undue experimentation factors of In re Wands, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) are set forth at MPEP §2164.01(a). These factors include:

- (a) the breadth of the claims;
- (b) the nature of the invention;
- (c) the state of the prior art;
- (d) the level of one of ordinary skill;
- (e) the level of predictability in the art;
- (f) the amount of direction provided by the

inventor;

- (g) the existence of working examples; and
- (h) the quantity of experimentation needed to make or use the invention based on the content of the disclosure.

Here, the examiner takes the position that the scope of the claims is broader than the enabled disclosure with respect to muteins of a leptin, with respect to fragments of leptin, and with respect to leptin receptor agonists. Before discussing enablement for the entire breadth of claim 28, the narrower issue of enablement of the dependent claims will

first be discussed. As indicated above, each of claims 30, 31, 32, 33, 34, 36, 37, 38, and 39 are patentable in their own right and must be considered independently for compliance with the enablement requirement of 35 USC 112. If each of these claims is considered to be based on an enabling disclosure, then claim 28, which is cumulative thereto, must also be considered to satisfy the enablement requirement. Claim 39 will be discussed first.

Claim 39 is directed to a method of claim 28 in which:

said active agent comprises a mutein of leptin having at least 90% identity with the sequence of a leptin and has the ability to block cell proliferation.

By emphasizing that the rejection is an enablement rejection and not based on the written description requirement, the examiner has effectively conceded that the present inventors were in the possession of all of the muteins which fall within this definition. Thus, the examiner effectively concedes that applicant was in possession of the necessary common attributes possessed by members of the genus, particularly since the claim requires that each member have the attribute of the ability to block cell proliferation.

With respect to the breadth of claim 39, this claim is broader than the use of leptin. The claimed scope is necessary in order to reasonably cover the invention. In MPEP

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Section 2164.08 relating to enablement commensurate in scope with the claims, the following is quoted from In re Goffe, 191 USPQ 429, 431 (CCPA 1976):

[T]o provide effective incentives, claims must adequately protect inventors. To demand that the first to disclose shall limit his claims to what he has found will work or to materials which meet the guidelines specified for "preferred" materials in a process such as the one herein involved would not serve the constitutional purpose of promoting progress in the useful arts.

In view of the stated activity and the direction in the specification, which will be discussed below, the breadth is not unduly broad and the experimentation to find everything within the scope of this claim would not be undue.

As to the nature of the invention, this is a therapeutic method and applicant concedes that there is not 100% predictability in this field. However, this does not mean that an applicant must be limited to exemplified embodiments. As long as it is shown that the experimentation to determine what falls within the claim is not undue, the enablement requirement is met. As discussed below, the experimentation is not undue.

As to the state of the prior art, there is no close prior art. The reference cited by the examiner here is not available as a reference because of its date. Thus, there is no prior art reason for limiting the scope of the claims.

As to the level of one of ordinary skill, therapeutic inventions, and particularly ones involving biotechnology, involve a very high level of ordinary skill. Because of this extremely high level of ordinary skill, even complex experimentation is not necessarily undue or unreasonable.

The next two Wands factors, the level of the predictability in the art and the amount of direction provided by the inventor, go hand in hand. As discussed above, the examiner is correct that it may not be entirely predictable what specific changes to the protein might entail insofar as the properties of that protein are concerned. However, the present claim always requires that the result of the mutation have the ability to block cell proliferation, i.e., by definition, the activity must be retained. The present specification states at page 8, lines 27-31:

Thus, it can be determined whether any given mutein has substantially the same activity as leptin by means of routine experimentation comprising subjecting such a mutein, e.g., to a simple cell proliferation assay, as a mutein which blocks cell proliferation retains sufficient activity of leptin and therefore has at least one of the disclosed utilities of leptin and thus has substantially similar activity thereto.

Furthermore, substantial guidance is provided in the present specification as to preferred substitutions which would be expected to retain the activity of the base compound,

i.e., leptin. Note, for example, page 9, line 4, through page 13, line 7. The examples in the present specification, such as examples 1-4, show well-known cell proliferation assays. Indeed, four different cell proliferation assays are shown. These are simple tests which may be done in 96-well plates so that many experiments can be done at one time. Accordingly, it is apparent that there is substantial direction provided in the specification about how to do these simple cell proliferation assays. This is all that is necessary to do in order to determine whether any given mutein having at least 90% identity with the sequence of a leptin has the ability to block cell proliferation. Accordingly, substantial direction is provided by the specification.

As far as working examples are concerned, as discussed above, many working examples of cell proliferation assays are given in the specification and the effect of leptin in these assays is provided in working examples. While there are no working examples given in the specification for muteins having at least 90% identity to leptin, the guidance of the specification explains how to determine whether any given compound falls within the scope of the claims, and therefore additional working examples are not necessary.

Finally, the last Wands factor is the quantity of experimentation needed to make or use the invention based on

the content of the disclosure. It is true that substantial experimentation may be necessary. However, as stated at MPEP §2164.06, the test is not merely quantitative since a considerable amount of experimentation is permissible if it is merely routine or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. Time and expense are not the controlling factors.

Procedures for making variants of leptin which have at least 90% identity with the sequence of leptin are conventional in the art. The assays involved to determine whether any such mutein has the ability to block cell proliferation are routine, as is disclosed in the specification and discussed above. All of the claimed muteins must possess the specified activity of being able to block cell proliferation. There is a reduction to practice of the single disclosed species of leptin. The fact that any single amino acid change could conceivably have a profound effect is not dispositive of the predictability issue. The fact is that one random point mutation, for example, while it could affect the ability to block cell proliferation, would not be predicted to do so. A single such random mutation would be expected to be innocuous.

Similarly, notwithstanding the above, it is reasonably predictable that muteins having such a relatively small amount of variation (no more than 10%), would retain the ability of leptin to block cell proliferation. But regardless of such predictability, the simple and rapidly performed assays disclosed in the specification can be conducted to establish that the ability to block cell proliferation has not been lost. A simple cell proliferation assay is provided in the specification and so any given mutein can readily be tested without undue experimentation. Thus, it is reasonable to predict that any mutein with 90% identity would retain the ability of leptin to block cell proliferation. Nevertheless, it is a routine matter to check this and discard any such muteins which lose such ability. Those that lose such ability would not be covered by the claims. Thus, the combination of reasonable predictability and the ability to test in the simple assay described in the specification that can be carried out in large numbers at the same time, establish that the specification contains sufficient information as to enable one skilled in the art to make and use the invention claimed in claim 39.

The level of skill in the art is high and the assay is simple and can be conducted with many different mutein sequences at the same time. Thus, while substantial

experimentation may be needed to establish that any given sequence falls within the scope of the claim, i.e., meets the functional requirement of blocking cell proliferation, such experimentation is not undue or unreasonable.

For all of these reasons, reversal of the examiner and withdrawal of this rejection with respect to the full scope of claim 39 are respectfully urged.

It Would Not Take Undue Experimentation to Practice the Full Scope of the Invention Claimed in Any One of Claims 38, 37 or 30

Claim 38 is identical to claim 39, discussed in the previous section, except that it requires that the mutein of leptin have at least 80% identity with the sequence of leptin as opposed to the 90% required by claim 39. Claim 37 is similarly identical except specifying at least 70% identity; and claim 30 is similarly identical except specifying at least 60% identity. If claim 39 is found to be enabled, as urged in the previous section, then claim 38 should also be enabled because approximately the same amount of additional experimentation as has been previously conducted to determine 90% identity can also reasonably be conducted in order to determine whether muteins of 80% identity retain the ability to block cell proliferation. Admittedly, the less identity, the greater the unpredictability as to whether or not a given sequence will retain the property of blocking cell

proliferation. Regardless of such predictability, however, simple cell proliferation assays are provided in the specification, which can be performed in mass and so large sets of any given muteins can readily be tested without undue experimentation. Thus, applicant needs not rely upon predictability with respect to changes.

Whether or not a given sequence would predictably block cell proliferation, the property of being able to block cell proliferation can be readily tested in the simple assays described in the specification which can be carried out in large numbers at the same time. Thus, the experimentation needed to practice the invention is neither undue nor unreasonable. The applicable Wands factors would therefore militate in favor of a finding that the use of a mutein with 80% identity, as in claim 38, is supported by an enabling disclosure. Similarly, the use of a mutein with 70% identity, as is claimed in claim 37, is also supported by an enabling disclosure, as is a mutein with 60% identity, as set forth in claim 30. Reversal of the examiner and withdrawal of this rejection in so far as claims 30, 38 and 37 are therefore also respectfully urged.

It Would Not Take Undue Experimentation to Practice the Full
Scope of the Invention Claimed in claim 31

As with claims 39, 38, 37, and 30 as discussed hereinabove, claim 31 is directed to a method in which the active agent is a mutein of leptin. However, in claim 31 the mutein is defined by the ability of the mutein to be encoded by a nucleic acid that hybridizes to a nucleic acid which encodes leptin under stringent conditions and has the ability to block cell proliferation. The conditions required for stringent hybridization are specifically defined in the present specification at page 13, lines 8-23. It should be noted that this paragraph refers to Sambrook et al (1989), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (see page 22, lines 27-28). Attached hereto as Exhibit A are pages 9.47-9.51 of this Sambrook publication referred to in the present specification. Reference is made to page 9.51 where it states:

b. T_m of a double-stranded DNA decreases by 1-1.5°C with every 1% decrease in homology (Bonner et al. 1973 ["Reduction in the Rate of DNA Reassociation by Sequence Diversions", J. Mol. Biol. 81:123]).

As the definition of stringent conditions in the above-cited portion of the specification includes "washing conditions 12-20°C below the calculated T_m of the hybrid under study" (page 13, lines 16-17), this should approximately correspond to an identity of at least 70%. Thus, if enablement is found in the

specification for claim 37, as discussed above, then enablement should also be found for claim 31.

It should be noted that it is not uncommon to claim muteins by such hybridization language. In this regard, reference is made to the Revised Interim Written Description Guidelines Training Materials, example 9, "Hybridization". In that example, the following claim was analyzed for compliance with the written description requirement of 35 USC 112:

An isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO:1, wherein said nucleic acid encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity.

The result of this analysis was that the claimed invention is adequately described. As the polypeptide encoded by any such nucleic acid hybridizing under the specified conditions can readily be tested without undue experimentation for ability to block cell proliferation, the experimentation needed to practice the invention is not undue or unreasonable. Accordingly, claim 31 should also be found to fully comply with the enablement requirement of 35 USC 112, first paragraph.

It Would Not Take Undue Experimentation To Practice the Full
Scope of the Invention Claimed in Claims 33 and 32

Claim 33 is directed to the same method as discussed in the above sections of this brief, but in which the active agent is a fragment of leptin. Claim 32 is directed to the same method as discussed in the above sections of this brief, but in which the active agent is a fragment of leptin or a fragment of a mutein as described in paragraph (b) of claim 28, and which has the ability to block cell proliferation. Paragraph (b) of claim 28 is cumulative of the subject matter of claims 30 and 31 discussed above.

With respect to claim 33, drawn to the use of fragments of leptin, the examiner has conceded that the use of the leptin of paragraph (a) is supported by enabling disclosure in view of the examiner's allowance of claim 9. Those of skill in the art well understand that fragments of leptin can be made by removing one amino acid at a time from either end and testing for activity using the simple assays described in the specification and discussed above. Once the activity is lost, it would not be expected that smaller fragments would be operable. Thus, the amount of experimentation needed to find fragments is even less than that needed to find muteins. It would not be expected that removal of a single N-terminal or C-terminal amino acid residue from a lengthy protein would affect the biological

activity of that protein. Thus, each of those fragments obtained by removing an amino acid one at a time from either end of the protein would be reasonably predictable to be active and this presumption could be readily tested by the simple assays which can be performed in mass as described in the present specification. However, once the amino acid is found which causes the fragment to lose activity, it would not be required to check even smaller fragments. The examiner has not explained why this testing of fragments would involve undue experimentation. The permutations are much smaller than those involved in the substitution of amino acids in the middle of the protein. Thus, fragments of leptin are surely based on an enabling disclosure.

As to claim 32, drawn to the use of fragments of leptin or fragments of the muteins, this part of the brief is contingent upon this Board finding that the muteins of claims 30 and 31 have been found to comply with the enablement requirement of the first paragraph of 35 USC 112 for the reasons presented hereinabove. Of course, if the muteins of paragraph (b) of claim 28 are not based on an enabling disclosure, then neither will fragments of those muteins. However, if they are considered to be enabled, then the fragments thereof must also be considered to be enabled for the same reasons as discussed above as to why fragments of

leptin would be considered to be enabled. For any given mutein which is found to be enabled, it would not take undue experimentation to determine which fragments thereof also retain the activity.

Accordingly, reversal of the examiner and withdrawal of the rejection of claims 33 and 32 are also respectfully urged.

It Would Not Take Undue Experimentation to Practice the Full Scope of the Invention Claimed in Claim 34

Claim 34 is drawn to the same method as the other claims discussed above except specifying that the active agent is a fusion protein comprising an agent of paragraphs (a), (b) or (c) of claim 28. The examiner has conceded by the allowance of claim 35 that if the use of the agent is enabled, then the use of a fusion protein comprising that agent is enabled. Claims 30, 31 and 32 are cumulatively the same as paragraphs (a), (b) and (c) of claim 28. Thus, if this Board finds that all of claims 30, 31 and 32 are supported by an enabling disclosure, then, in view of the examiners admission about fusion proteins, claim 34 must also be supported by an enabling disclosure. e

Accordingly, reversal of the examiner and withdrawal of the rejection of claim 34 are also respectfully urged.

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It Would Not Take Undue Experimentation To Practice the Full
Scope of the Invention Claimed in Claim 36

Claim 36 is drawn to the same method as the other claims discussed above except specifying that the active agent is a leptin receptor agonist which has the ability to block cell proliferation. In the final rejection of July 30, 2002, the examiner states that the receptor agonists need not share structural similarities and thus it is not possible to predict the structural requirement necessary to both bind to leptin receptor and block cell proliferation. This is correct. However, it is not necessary to be able to predict the structure of an agent that will work in the method of the present invention in order for there to be an enabling disclosure. As long as the disclosure discloses how to obtain the agonists which may be used in the method of the present invention without undue experimentation, then the enablement requirement is satisfied. As discussed hereinabove, the question is whether the experimentation needed to practice the invention is undue or unreasonable.

Leptin receptor agonists are discussed in the paragraph bridging pages 14 and 15 of the present specification. The specification states that such agonists may be selected from a library of peptides, a library of peptide analogs or a random library of organic molecules by means known in the art, i.e., by the ability of the selected

agonists to bind to the leptin receptor. Thus, a population of agents that bind to the leptin receptor, can be found without undue experimentation by means of a simple binding assay with the leptin receptor which can be done in mass using libraries of such agents which are readily available. Methods of doing this, for example by phage display, are described in the paragraph bridging pages 14 and 15 of the present specification. In the last sentence of that paragraph, the specification explains that candidate peptides, identified by their affinity for the leptin receptor, are then further selected by their ability to inhibit cell proliferation in the aforementioned manner.

Accordingly, it is irrelevant that the structure of these agonists are unknown *ab initio*. The specification explains that they can be found without undue experimentation by screening peptide libraries or organic molecule libraries for binding to the leptin receptor by any of the procedures discussed in the specification. Those that bind to the leptin receptor are then tested for blocking cell proliferation in the assays discussed hereinabove.

The examiner has not explained why it is considered to be undue experimentation to find operable receptor agonists. Cases such as In re Wands, supra, do not require that the structure be known in advance, indeed Wands is an

antibody case, and the sequence of the antibodies which may be found by the screen disclosed therein would not have been predictable *ab initio*. The present invention is directed to a novel method, not to agents *per se*. At least one such receptor agonist is disclosed in the present specification, i.e., leptin. The examiner conceded written description for the receptor agonists being claimed. Strictly considering the issue of enablement, such agonists can be found without undue experimentation for the reasons discussed above.

Accordingly, reversal of the examiner and withdrawal of the rejection with respect to claim 36 is hereby respectfully urged.

It Would Not Take Undue Experimentation To Practice the Full Scope of the Invention Claimed in Claims 28 and 2-8

Claim 28 is cumulative to claims 30, 31, 32, 34, and 36 in its paragraphs (a) to (e). Thus, if enablement is found for all of these claims for the reasons discussed above, then 28 is also based on enabling disclosure, at least for paragraphs (a) to (e). Paragraph (f) of claim 28 is drawn to a salt or functional derivative of any of (a) to (e). Salts and functional derivatives are defined at page 13, line 28, to page 14, line 20, of the present specification. There is no reason to believe that simple salts of the carboxy groups or acid addition salts of the amino groups of leptin will affect

the activity thereof. Typically, salts are made in order to modify pharmacological properties, not pharmaceutical properties.

Functional derivatives, by the definition in the present specification, do not destroy the activity of the protein and do not confer toxic properties on it. Again, these are mere derivatives of the functional groups which occur as side chains on the residues or the N- or C-terminal groups. Such derivatives are well known for improving pharmacological properties such as solubility, *in vivo* half life, etc. There is no reason to expect that they would affect the ability to block cell proliferation, but if such should happen, it would not be included in the claim because, by definition, a functional derivative must have an activity substantially similar to the activity of leptin, i.e., the ability to block cell proliferation as well as not being toxic. Accordingly, the examiner has not established a *prima facie* case that the salts or functional derivatives of section (f) are not supported by an enabling disclosure. For all of the reasons discussed in previous sections as well as in this section of the brief, claim 28 is supported by an enabling disclosure.

The examiner has specifically relied on Bjorbaek et al to support the enablement rejection. The examiner relies

on Bjorbaek for its teaching that, for the leptin to exert its effect of activating² the phosphorylation of IRS-1, it must bind to the leptin receptor to activate JAK-2. However, this finding is diametrically opposed to the finding of the present invention. The present invention is directed to the inhibition of phosphorylation of IRS-1, while Bjorbaek relates to the activation of IRS-1. In this regard, reference is made to page 1, lines 29-31, of the present specification, relating to the effect of leptin on IRS-1 phosphorylation and citing Cohen et al, "Modulation of Insulin Activities By Leptin", Science, 274:1185-1188 (1996). A copy of this publication is attached hereto as Exhibit B. This publication relates to tests on actual carcinoma cell lines showing that leptin inhibited tyrosine phosphorylation of IRS-1. Bjorbaek even cites and discusses this reference at the second column of 32693, last full paragraph, conceding that they could not explain the apparent discordance between the two findings. However, in view of the fact that the experimentation in the present specification is done on actual cancer cells and not embryonic cells to which IRS-1 and leptin receptor are added

² The examiner incorrectly characterizes Bjorbaek as relating to the inhibition of the phosphorylation of IRS-1. As discussed above in the section describing the prior art, Bjorbaek clearly relates to activation of IRS-1, not inhibition (see section bridging 32690 and 32691 and first full paragraph of the second column of 32693).

by genetic transformation, there is no reason to doubt the accuracy of the teachings in the present specification.

Furthermore, the present claims do not require that there be a binding to any type of receptor or substrate. The test for determining whether the leptin muteins, fragments, or leptin receptor agonists fall within the scope of the claim is a tumor cell proliferation assay. It is not necessary to know whether JAK-2 will be activated, or anything else about the theory as to why the present invention works. It is only necessary to identify the fragments, muteins or agonists, and then test for ability to block cell proliferation in any of the simple assays described in the specification. If this does not entail undue or unreasonable experimentation, then the claims are based on an enabling disclosure, notwithstanding anything that is disclosed by Bjorbaek.

For all of these reasons, reversal of the examiner and allowance of claim 28, and all of the other claims subject to the present rejection are respectfully urged.

CONCLUSION

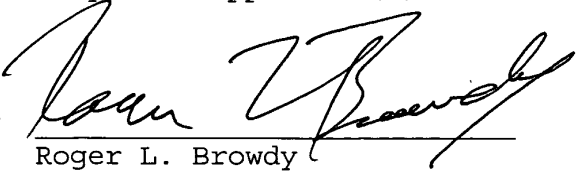
The claims as submitted are believed to truly set forth the inventive concept of the present invention and to fully comply with the enablement requirement of the first paragraph of 35 USC 112. Accordingly, reversal of the

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examiner and allowance of all of claims 2-8, 28, 30-34 and 36-39 are earnestly solicited.

Respectfully submitted,

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APPENDIX A

2. The method according to claim 28 for inhibiting cell proliferation for the treatment of malignancies in mammals.

3. The method according to claim 28 for inhibiting growth-factor dependent tumors.

4. The method according to claim 28 for inhibiting human breast carcinoma cell proliferation.

5. The method according to claim 4 for treatment of human breast carcinomas.

6. The method according to claim 28 for inhibiting the growth stimulatory effect of insulin on tumor cells, as mediated, at least partially, by the insulin receptor substrate-1 (IRS-1)/growth-factor receptor-associated binding protein-2 (GRB2) pathway.

7. The method according to claim 28 for inhibiting the mitogenic responses in tumor cells to one or more receptor kinases, growth factors and cytokines of the group consisting of IGF-1, IL-4 and IL-9, for all of which IRS-1 is a substrate, for the treatment of tumors.

8. The method according to claim 28 for inhibiting basal and insulin-induced tumor cell proliferation for the treatment of human breast cancers.

28. A method for treating tumors in mammals or for inhibiting tumor cell proliferation in mammals, comprising administering to a mammal in need thereof an effective amount of an active agent selected from the group consisting of:

(a) leptin;

(b) a mutein of leptin having at least 60% identity with the sequence of a leptin and has the ability to block cell proliferation or having a sequence encoded by a nucleic acid which hybridizes to a nucleic acid which encodes leptin under stringent conditions and has the ability to block cell proliferation;

(c) a fragment of one of (a) or (b) which has the ability to block cell proliferation;

(d) a fusion protein comprising (a), (b) or (c);

(e) a leptin receptor agonist which has the ability to block cell proliferation; and

(f) a salt or functional derivative of any of (a)-(e).

30. A method in accordance with claim 28, wherein said active agent is a mutein of leptin having at least 60% identity with the sequence of a leptin and has the ability to block cell proliferation.

31. A method in accordance with claim 28, wherein said active agent is a mutein of leptin having a sequence

encoded by a nucleic acid which hybridizes to a nucleic acid which encodes leptin under stringent conditions and has the ability to block cell proliferation.

32. A method in accordance with claim 28, wherein said active agent is a fragment of (a) or (b) of claim 28, which has the ability to block cell proliferation.

33. A method in accordance with claim 32, wherein said active agent is a fragment of leptin which has the ability to block cell proliferation.

34. A method in accordance with claim 28, wherein said active agent is a fusion protein comprising (a), (b) or (c) of claim 28.

36. A method in accordance with claim 28, wherein said active agent is a leptin receptor agonist which has the ability to block cell proliferation.

37. A method in accordance with claim 28, wherein said active agent comprises a mutein of leptin having at least 70% identity with the sequence of a leptin and has the ability to block cell proliferation.

38. A method in accordance with claim 28, wherein said active agent comprises a mutein of leptin having at least 80% identity with the sequence of a leptin and has the ability to block cell proliferation.

In re of Appln. No. 09/403,897

39. A method in accordance with claim 28, wherein said active agent comprises a mutein of leptin having at least 90% identity with the sequence of a leptin and has the ability to block cell proliferation.

APPENDIX B

9. The method according to claim 28, wherein said active ingredient is leptin, and said leptin is used as said inhibitor or for said treatment.

29. A method in accordance with claim 28, wherein said active agent is leptin.

35. A method in accordance with claim 34, wherein said active agent is a fusion protein comprising leptin.

2

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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HYBRIDIZATION OF RADIOLABELED PROBES TO IMMOBILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)
- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)
- Degree and method of agitation (continuous shaking or stationary)
- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix
- Concentration of the labeled probe and its specific activity
- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or polyethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids
- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.
2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.
3. Continual movement of the probe solution across the filter is unnecessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneously, agitation is advisable to prevent the filters from adhering to one another.
4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the

immobilized nucleic acid and its availability for hybridization are unknown. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1-3 \times C_0 t_{1/2}$. In 10 ml of hybridization solution, 1 μg of a probe of 5-kb complexity will reach $C_0 t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_0 t_{1/2}$$

where x = the weight of the probe added (in micrograms), y = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and z = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_0 t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by prehybridizing filters with a blocking agent consisting of $5 \times$ Denhardt's reagent, 0.5% SDS, and 100 $\mu\text{g}/\text{ml}$ denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/Hogness hybridization [1975], Benton/Davis hybridization [1977], Southern hybridization [1975] of abundant DNA sequences, etc.), we recommend using 0.25% nonfat dried milk ($0.05 \times$ BLOTTO; Johnson et al. 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfactory. In general, Denhardt's reagent is more effective for nylon membranes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.
6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However, when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

TABLE 9.1 Blocking Agents Used to Suppress Background in Hybridization Experiments

Ag nt	Recommended uses
Denhardt's reagent	northern hybridizations hybridizations using RNA probes single-copy Southern hybridizations hybridizations involving DNA immobilized on nylon membranes
Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at -20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 µg/ml denatured, fragmented, salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia) 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Pentex Fraction V), and H ₂ O to 500 ml.	
BLOTTO	Grunstein/Hogness hybridization Benton/Davis hybridization all Southern hybridizations other than single-copy dot blots

1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% non-fat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into hybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

Heparin	Southern hybridization in situ hybridization
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Heparin (Sigma H-7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 µg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 µg/ml (Singh and Jones, 1984).

Denatured, fragmented salmon sperm DNA	Southern and northern hybridizations
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Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2-4 hours at room temperature to help the DNA to dissolve. The solution is adjusted to 0.1 M NaCl and extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered and the DNA is sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation and redissolved at a concentration of 10 mg/ml in water. The OD₂₆₀ of the solution is determined and the exact concentration of the DNA is calculated. The solution is then boiled for 10 minutes and stored at -20°C in small aliquots. Just before use, the solution is heated for 5 minutes in a boiling-water bath and then chilled quickly in ice water. Denatured, fragmented salmon sperm DNA should be used at a concentration of 100 µg/ml in hybridization solutions.

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.
8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength ($6 \times$ SSC or $6 \times$ SSPE) at a temperature that is $20\text{--}25^\circ\text{C}$ below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, $6 \times$ SSPE is preferred because of its greater buffering power.
9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately $12\text{--}20^\circ\text{C}$ below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.
10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains $10\text{ }\mu\text{g}$ of DNA, $10\text{--}20\text{ ng/ml}$ radiolabeled probe (sp. act. = 10^9 cpm/ μg or greater) should be used and hybridization should be carried out for $12\text{--}16$ hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for $6\text{--}8$ hours using $1\text{--}2\text{ ng/ml}$ radiolabeled probe (sp. act. = 10^9 cpm/ μg or greater).
11. *Useful facts:*
 - a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

$$T_m = 81.5^\circ\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{ G} + \text{C}) - 0.63(\% \text{ formamide}) - (600/l)$$

where l = the length of the hybrid in base pairs.

This equation is valid for:

- Concentrations of Na^+ in the range of 0.01 M to 0.4 M. It predicts T_m less accurately in solutions of higher $[\text{Na}^+]$.
- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of T_m in solutions containing formamide is greater for poly(dA:dT) (0.75°C/1% formamide) and less for DNAs rich in poly(dG:dC) (0.50°C/1% formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" T_m that is defined by optical measurement of hyperchromicity at OD_{257} . The "irreversible" T_m , which is more important for autoradiographic detection of DNA hybrids, is usually 7–10°C higher than that predicted by the equation.

Similar equations have been derived for:

- i. RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_m = 79.8^\circ\text{C} + 18.5(\log_{10}[\text{Na}^+]) + 0.58(\% \text{ G} + \text{C}) + 11.8(\% \text{ G} + \text{C})^2 - 0.35(\% \text{ formamide}) - (820/l)$$

- ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8^\circ\text{C} + 18.5(\log_{10}[\text{Na}^+]) + 0.58(\% \text{ G} + \text{C}) + 11.8(\% \text{ G} + \text{C})^2 - 0.50(\% \text{ formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the T_m of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the T_m of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

- b. The T_m of a double-stranded DNA decreases by 1–1.5°C with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

at the highest E fields, thus permitting the use of thicker samples.

We have measured data storage error rates in a holographic optical storage test stand (27–29). Here a coherent 676-nm, data-containing object beam is intersected with a plane-wave reference beam in the PR sample (90% 2BNCM, 9.7% PMMA, 0.3% TNF) and a hologram is formed. A portion of a 64-kbit random data page is shown in Fig. 2. The size of each data bit on the mask was 18 μm by 18 μm , and each bit was surrounded by a 9 μm thick, opaque boarder with an overall pitch of 36 μm by 36 μm . The coherent object beam is passed through the data page mask containing the information as just described and focused on the sample to a 4-mm-diameter spot. The hologram is read back by illuminating the sample with only the reference beam and the reconstructed hologram recorded on a charge-coupled device (CCD) detector array. Experiments involved the storage, retrieval, and subsequent erasure of digital data pages with a data density of 0.5 Mbit/cm². For the amorphous glass system described here, single data pages could be stored and retrieved without error using only 1 part in 10⁶ of the total dynamic recording range (that is, Δn range). Holograms were read back periodically at room temperature in air for up to 6 hours with no observable degradation in hologram quality or bit error rate.

The vanishingly small scattering levels in these glasses reduce the hologram efficiency required for error-free readout by a factor of 10 compared with polymeric systems studied previously. Of perhaps equal importance is the extremely large recording dynamic range available in these dihydropyridine systems. The overmodulation of the holographic efficiency, occurring at relatively small E fields (see Fig. 1), means that the smaller field can be applied to thicker samples, increasing the Bragg sensitivity, the number of holograms that can be multiplexed, and the overall hologram quality. In relation to existing polymeric PR systems, these organic glasses would appear to offer substantial advantages.

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Modulation of Insulin Activities by Leptin

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Leptin mediates its effects on food intake through the hypothalamic form of its receptor OB-R. Variants of OB-R are found in other tissues, but their function is unknown. Here, an OB-R variant was found in human hepatic cells. Exposure of these cells to leptin, at concentrations comparable with those present in obese individuals, caused attenuation of several insulin-induced activities, including tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1), association of the adapter molecule growth factor receptor-bound protein 2 with IRS-1, and down-regulation of gluconeogenesis. In contrast, leptin increased the activity of IRS-1-associated phosphatidylinositol 3-kinase. These *in vitro* studies raise the possibility that leptin modulates insulin activities in obese individuals.

Leptin, an adipocyte-derived cytokine that regulates body weight, was identified by positional cloning of the murine *obese (ob)* gene (1) and was shown to affect both food intake and thermogenesis (2). High-affinity leptin-binding sites were detected in the choroid plexus, which led to identification of the leptin receptor OB-R (3). The known activities of leptin are mediated through the hypothalamic OB-R, but OB-R and OB-R variants derived from alternative splicing are expressed in other tissues, notably the kidney, lung, and liver (3–5). This receptor expression pattern suggests that, in addition to control of food intake and body heat, leptin may have other physiological functions. Although leptin is produced by adipocytes, the recent finding that excess fat correlates with high concentrations of leptin in serum (6), and the well-established linkage between obesity and insulin

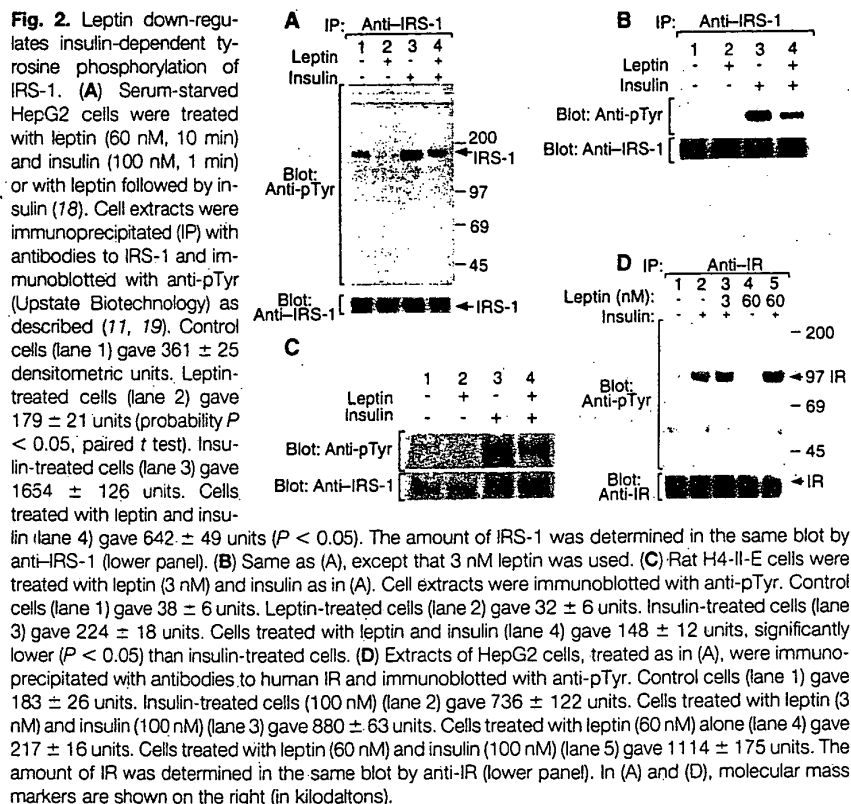
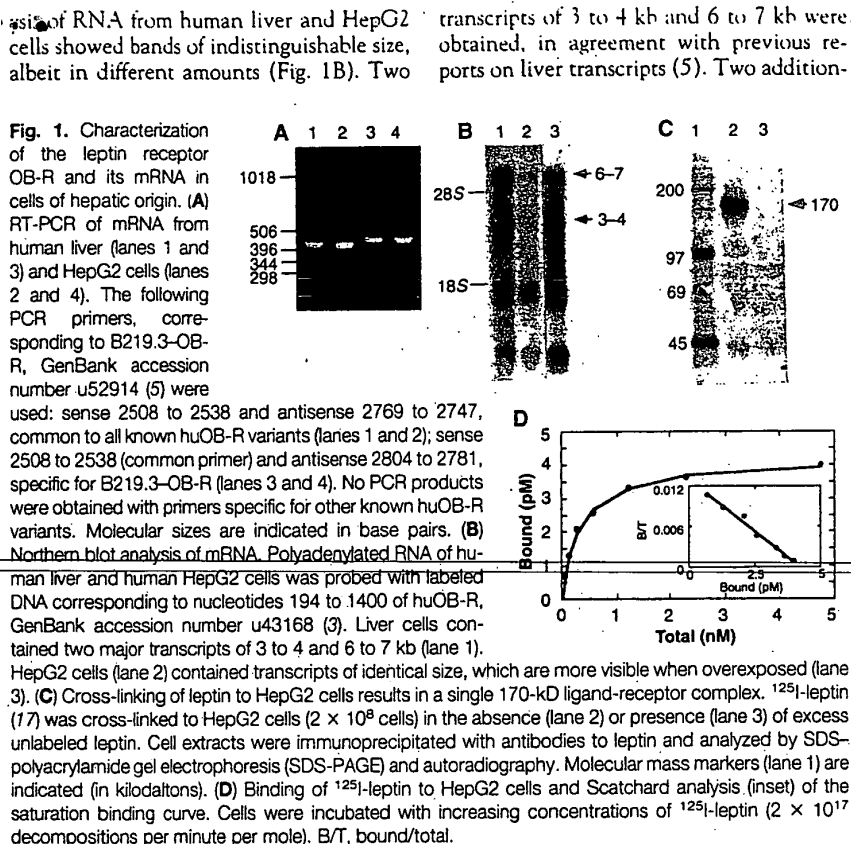
resistance (7), led us to explore the possibility that leptin may modulate insulin-regulated responses.

To test for possible effects of leptin on insulin-regulated responses, we looked for cell lines expressing a functional OB-R. Various human cell lines derived from liver, lung, and kidney were screened by reverse transcription-polymerase chain reaction (RT-PCR) with oligonucleotides corresponding to the region encoding the extracellular domain of human OB-R (huOB-R) (3). The human hepatocellular carcinoma cell lines HepG2 and Hep3B provided one PCR product whose identity with huOB-R mRNA was confirmed by DNA sequencing. Additional RT-PCR was done with primers corresponding to specific 3' end regions of the four known splice variants of huOB-R. Only one splice variant, with a short cytoplasmic domain (the B219.3-OB-R mRNA) (5), was detected in HepG2 cells. The same product was obtained when RT-PCR was done with mRNA from human liver (Fig. 1A), and its identity was confirmed by sequencing. Northern blot anal-

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Fig. 1. Characterization of the leptin receptor OB-R and its mRNA in cells of hepatic origin. (A) RT-PCR of mRNA from human liver (lanes 1 and 3) and HepG2 cells (lanes 2 and 4). The following PCR primers, corresponding to B219.3-OB-R, GenBank accession number u52914 (5) were used: sense 2508 to 2538 and antisense 2769 to 2747, common to all known huOB-R variants (lanes 1 and 2); sense 2508 to 2538 (common primer) and antisense 2804 to 2781, specific for B219.3-OB-R (lanes 3 and 4). No PCR products were obtained with primers specific for other known huOB-R variants. Molecular sizes are indicated in base pairs. (B) Northern blot analysis of mRNA. Polyadenylated RNA of human liver and human HepG2 cells was probed with labeled DNA corresponding to nucleotides 194 to 1400 of huOB-R, GenBank accession number u43168 (3). Liver cells contained two major transcripts of 3 to 4 and 6 to 7 kb (lane 1). HepG2 cells (lane 2) contained transcripts of identical size, which are more visible when overexposed (lane 3). (C) Cross-linking of leptin to HepG2 cells in a single 170-kD ligand-receptor complex. 125 I-leptin (17) was cross-linked to HepG2 cells (2×10^6 cells) in the absence (lane 2) or presence (lane 3) of excess unlabeled leptin. Cell extracts were immunoprecipitated with antibodies to leptin and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Molecular mass markers (lane 1) are indicated (in kilodaltons). (D) Binding of 125 I-leptin to HepG2 cells and Scatchard analysis (inset) of the saturation binding curve. Cells were incubated with increasing concentrations of 125 I-leptin (2×10^{17} decompositions per minute per mole). B/T, bound/total.



al RNA molecules were detected, but they were too short to encode a transmembrane receptor.

At the protein level, ligand-binding experiments revealed the presence of a functional OB-R in cells of hepatic origin. Cross-linking of 125 I-labeled leptin (15 kD) to HepG2 cells, followed by immunoprecipitation with antibodies to leptin, yielded a single 170-kD band, corresponding to the receptor-ligand complex (Fig. 1C). HepG2 cells bound 125 I-leptin in a saturable manner, and Scatchard analysis of the binding data revealed one class of high-affinity binding sites (5000 ± 400 sites per cell), with a dissociation constant (K_d) of 0.32 ± 0.038 nM (Fig. 1D). This K_d value is consistent with that reported for transfected cells expressing the hypothalamic OB-R (3). 125 I-leptin bound specifically to other cell types, including the rat hepatoma cell line H4-II-E, as determined by displacement with excess unlabeled ligand.

We next tested the effect of leptin on the profile of tyrosine-phosphorylated cellular proteins. HepG2 cells were treated with leptin for 5 to 30 min, and cellular proteins were analyzed by immunoblot analysis with antibodies to phosphotyrosine (anti-pTyr). The most profound effect of leptin was a reduction in the amount of a tyrosine-phosphorylated 185-kD protein, identified as the insulin receptor substrate-1 (IRS-1) by immunoprecipitation with a specific antibody. Pretreatment of HepG2 cells with leptin (3 or 60 nM) for 10 min down-regulated by a factor of 2 both the basal and the insulin-induced (100 nM, 1 min) tyrosine phosphorylation of IRS-1 (Fig. 2, A and B). Leptin reduced IRS-1 phosphorylation by a factor of 1.5 in H4-II-E cells (Fig. 2C). The effect of leptin on IRS-1 phosphorylation was specific, because tyrosine phosphorylation of the insulin receptor (IR) β chain was not reduced but rather was slightly increased (Fig. 2D).

Downstream signaling of IRS-1 is mediated by several associated proteins, including the adapter molecule growth factor receptor-bound protein 2 (GRB2) and phosphatidylinositol 3-kinase (PI 3-kinase) (8). We therefore tested the effect of leptin on the interaction of GRB2 and PI 3-kinase with IRS-1. Pretreatment of HepG2 cells with leptin reduced the basal and insulin-induced number of GRB2 binding sites in IRS-1 by factors of 1.4 and 1.8, respectively, as determined by the association of IRS-1 with a fusion protein consisting of glutathione-S-transferase (GST) and GRB2 (Fig. 3A). In addition, leptin reduced the basal and the insulin-induced binding of endogenous GRB2 to IRS-1 by a factor of 1.4, as determined by immunoprecipitation with anti-GRB2 and immunoblot analysis with

anti-IRS-1 (Fig. 3B). Similarly treated cells were analyzed for the association of PI 3-kinase with IRS-1. Here, both leptin and insulin increased the binding of PI 3-kinase to IRS-1 by threefold and fourfold, respectively, as determined by immunoprecipitation of cell extracts with antibodies to the p85 subunit of PI 3-kinase and immunoblot analysis with anti-IRS-1 (Fig. 4A). No additive increase of PI 3-kinase binding to IRS-1 was noticed when the cells were treated with both leptin and insulin (Fig. 4A). The leptin-induced increase in association of IRS-1 with PI 3-kinase was in apparent contrast with its attenuating effect on pTyr phosphorylation of IRS-1. We therefore measured the amount of PI 3-kinase catalytic activity in IRS-1 immunoprecipitates. Leptin and insulin increased the IRS-1-associated PI 3-kinase activity by twofold and fourfold, respectively, whereas a combination of the agents increased the activity by ninefold (Fig. 4B).

GRB2 and PI 3-kinase bind to different pTyr residues within IRS-1 (9). It is thus possible that most of the pTyr residues of IRS-1, including the GRB2 binding site (Tyr⁸⁹⁵), are dephosphorylated in response to leptin, whereas the PI 3-kinase binding site (either Tyr⁶⁰⁸ or Tyr⁹³⁹) may become phosphorylated. Such opposite effects of leptin may involve activation or recruitment of a specific protein tyrosine phosphatase and kinase. Alternatively, leptin may induce the phosphorylation of IRS-1 at serine residues, thereby inhibiting the IR kinase, as was reported in the case of tumor necrosis factor- α (TNF- α) (10). The effects of leptin on IRS-1 are not necessarily mediated through the IR kinase. Rather, they may result from an independent OB-R signaling cascade. Indeed, IRS-1 is not a specific substrate of the IR kinase. Rather, it is phosphorylated by additional growth factors and cytokines, including insulinlike growth factor-1, interferon- α , interleukin-4 (IL-4), and IL-9 (11, 12). The rather short cytoplasmic domain of OB-R suggests the involvement of an accessory receptor subunit. Alternatively, it is possible that signaling in HepG2 cells is mediated by a longer form of OB-R that has not yet been identified.

We also investigated whether leptin affects glucose homeostasis in cell culture. Hepatic and renal gluconeogenesis is a major factor in maintaining glucose homeostasis. The rate-limiting enzyme of gluconeogenesis is phosphoenolpyruvate carboxykinase (PEPCK). This enzyme has no known allosteric control and is down-regulated by insulin at the transcriptional level. The rat hepatoma cell line H4-II-E has been used successfully to study the regulation of PEPCK expression, whereas HepG2 cells do not express PEPCK efficiently (13). The

amount of PEPCK mRNA in H4-II-E cells treated first with $N^6,2'$ -O-dibutyryl adenosine 3',5'-monophosphate (dibutyryl

cAMP) and then with insulin was reduced by a factor of 4.8 compared to cells treated with dibutyryl cAMP alone. Incubation of

Fig. 3. Leptin attenuates the association of GRB2 with IRS-1. HepG2 cells were treated as in Fig. 2A. (A) Cell extracts were bound to GST-GRB2 fusion protein and glutathione-agarose, subjected to SDS-PAGE, and immunoblotted with anti-IRS-1 (20) followed by densitometry. Constitutive binding of IRS-1 to GST-GRB2 (257 ± 36 arbitrary units) was seen in control cells (lane 1). Leptin reduced this binding to 181 ± 23 units (lane 2). The number of GRB2 binding sites of IRS-1 in insulin-treated cells (626 ± 59 units, lane 3) was reduced to 353 ± 41 ($P < 0.02$) units upon treatment with leptin (lane 4). Molecular mass markers (lane 5) are indicated (in kilodaltons). The same amount of GST-GRB2 fusion protein was present in each lane, as shown by Ponceau S staining of the nitrocellulose membrane (lower panel). (B) Extracts were immunoprecipitated (IP) with polyclonal anti-GRB2 (Santa Cruz Biotechnology) and immunoblotted with anti-IRS-1 (upper panel). The amount of basal GRB2-associated IRS-1 (118 ± 16 units, lane 1) was decreased by leptin to 84 ± 12 units ($P < 0.08$, lane 2). The insulin-induced amount of GRB2-associated IRS-1 (327 ± 49 units, lane 4) was reduced to 228 ± 27 units ($P < 0.05$, lane 3). The amount of GRB2 was determined with anti-GRB2 (lower panel).

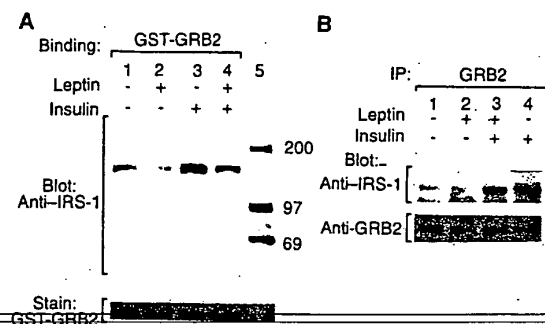


Fig. 4. Leptin increases the association of PI 3-kinase (PI 3-K) with IRS-1 and its activity. (A) HepG2 cells were treated as in Fig. 2A. Cell extracts were immunoprecipitated (IP) with polyclonal anti-PI 3-kinase (p85, Upstate Biotechnology) and immunoblotted with anti-IRS-1 (upper panel). The basal amount of PI 3-kinase, associated with IRS-1 (78 ± 7 units, lane 1), was increased both by leptin (240 ± 21 units, $P < 0.002$, lane 2) and by insulin (310 ± 28 units, $P < 0.001$, lane 3) as well as by treatment with both leptin and insulin (299 ± 26 units, lane 4). The amount of PI 3-kinase was determined with anti-PI 3-kinase (lower panel). Molecular mass markers are shown on the right (in kilodaltons). (B) HepG2 cells were treated as in Fig. 2A. Cell extracts were immunoprecipitated with polyclonal anti-IRS-1 and the immunoprecipitates were analyzed for in vitro PI 3-kinase activity (11). The basal level of IRS-1-associated PI 3-kinase activity, as determined by formation of phosphatidylinositol 4-phosphate (PIP; 740 ± 70 densitometric units, lane 1) was increased by leptin to 1583 ± 115 units ($P < 0.003$, lane 2) and by insulin to 2830 ± 231 units ($P < 0.005$, lane 3). Combined treatment with leptin and insulin increased the activity to 7030 ± 577 units ($P < 0.006$, lane 4).

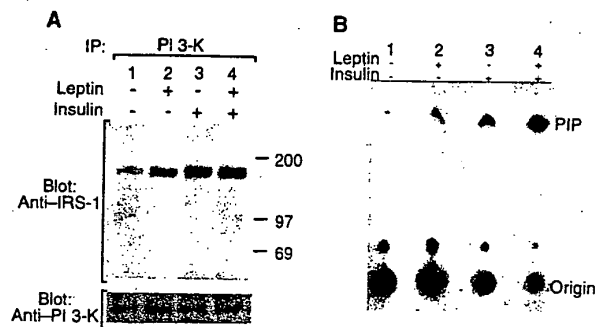
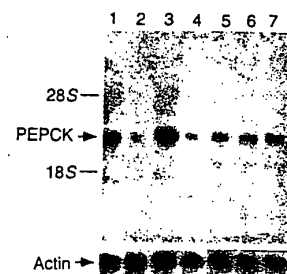


Fig. 5. Leptin up-regulates PEPCK expression. Serum-starved rat hepatoma H4-II-E cells (13) were pretreated with dibutyryl cAMP (0.5 mM, 3 hours) followed by treatment with leptin and insulin as in Fig. 2A. Northern blot analysis was done on cytoplasmic RNA with a DNA probe corresponding to rat PEPCK mRNA (positions 1364 to 1869 from the ATG start site). The amount of cytoplasmic PEPCK mRNA (arrow) in cells treated with insulin (10 nM, 2 hours) was reduced by a factor of 4.8 to 8.6 (333 ± 22 densitometric units, $P < 0.0001$, lane 2) compared with control cells or leptin-treated (60 nM, 2 hours) cells (1588 ± 121 and 2860 ± 244 units, lanes 1 and 3, respectively). Pretreatment of cells with leptin (3 nM) for 0.5, 1, or 2 hours, followed by treatment with insulin (10 nM, 2 hours), partially reversed the insulin-induced down-regulation of PEPCK mRNA expression (309 ± 21 units, lane 4; 480 ± 35 units, $P < 0.05$, lane 5; 585 ± 36 units, $P < 0.005$, lane 6). Pretreatment with 60 nM leptin for 1 hour, followed by treatment with insulin (10 nM, 2 hours) gave 741 ± 66 units ($P < 0.005$, lane 7). The same amount of RNA was present in each lane, as shown by reblotting the membrane with a probe corresponding to rat actin mRNA (positions 1670 to 2452 of the rat actin gene, lower panel).



the cells with leptin for 1 to 2 hours before addition of insulin partially reversed the down-regulating effect of insulin on PEPCK expression (Fig. 5). This observation is in contrast with the leptin-induced increase in PI 3-kinase activity obtained in HepG2 cells. Furthermore, the kinetics of the effect of leptin on PEPCK expression excludes its possible mediation by GRB2. It is therefore likely that leptin affects PEPCK gene expression by an as yet unknown pathway. On the basis of these results, we propose that leptin may affect gluconeogenesis, at least in the H4-II-E cell line, by attenuating the effect of insulin on the expression of PEPCK.

Several mouse strains that are deficient in leptin or OB-R serve as models for obesity, insulin resistance, and non-insulin-dependent diabetes mellitus (1, 4, 14). Therefore, it appears that excess leptin, as well as a complete absence of leptin, may impair some insulin responses, although not necessarily by the same mechanism. In the absence of leptin, other obesity-related factors may attenuate insulin responses. One such factor is TNF- α , which is overexpressed in adipocytes of obese animals. TNF- α down-regulates insulin-induced phosphorylation of IRS-1 and reduces expression of the insulin-dependent glucose transporter Glut4 (10, 15).

Tyrosine phosphorylation of IRS-1 by the IR kinase is a key step in the IR signaling cascade, and GRB2 further mediates parts of this cascade (8, 16). Therefore, the leptin-induced dephosphorylation of IRS-1 and its dissociation from GRB2 indicate that leptin may antagonize some functions of insulin. Although insulin resistance is poorly understood, it probably results from a combination of several factors and processes. Our finding that leptin attenuates some insulin-induced signals in hepatic cell lines, and the reports of increased serum leptin in obesity, warrants further studies on the possible role of leptin in obesity-associated insulin resistance.

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17. Mouse leptin was purchased from Peprotech (Princeton, NJ). Antiserum to mouse leptin was ob-

18. Serum-starved (24 hours) cells (4×10^7 cells) in Eagle's minimal essential medium were treated with insulin and leptin as indicated. Cells were washed three times with cold phosphate-buffered saline containing 1 mM Na₂VO₄ and lysed with 1 ml of cold lysis buffer [50 mM Tris-HCl (pH 8.0), 1% Triton X-100, 1 mM Na₂EDTA, 1 mM MgCl₂, 100 mM NaF, 1 mM phenylmethanesulfonyl fluoride, and aprotinin (5 μ g/ml)]. Immunoprecipitation or precipitation with GST-GRB2 fusion protein was done on the clarified lysates. All autoradiograms were quantitated by densitometry and are averages of three independent experiments.
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From Peptide Precursors to Oxazole and Thiazole-Containing Peptide Antibiotics: Microcin B17 Synthase

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Escherichia coli microcin B17 is a posttranslationally modified peptide that inhibits bacterial DNA gyrase. It contains four oxazole and four thiazole rings and is representative of a broad class of pharmaceutically important natural products with five-membered heterocycles derived from peptide precursors. An in vitro assay was developed to detect heterocycle formation, and an enzyme complex, microcin B17 synthase, was purified and found to contain three proteins, McbB, McbC, and McbD, that convert 14 residues into the eight mono- and bisheterocyclic moieties in vitro that confer antibiotic activity on mature microcin B17. These enzymatic reactions alter the peptide backbone connectivity. The propeptide region of premicrocin is the major recognition determinant for binding and downstream heterocycle formation by microcin B17 synthase. A general pathway for the enzymatic biosynthesis of these heterocycles is formulated.

A growing number of peptide-based natural products have been found to contain thiazole and oxazole heterocyclic rings and exhibit significant antifungal, antibiotic, antitumor, and antiviral biological activities (1). The five molecules depicted in Fig. 1, bleomycin A₂, thiagazole, patellamide A, pristinamycin II_A, and thiostrepton, exemplify the patterns of such heterocycle occurrence in molecules of therapeutic in-

terest. The antitumor antibiotic bleomycin uses the bithiazole moiety to intercalate into DNA (2). The tandem four-ring structure of thiagazole including the β -methyloxazoline provides potent antiviral activity against human immunodeficiency virus (3). Patellamides, cyclic octapeptides of marine origin, have antitumor properties (4). Thiostrepton, a protein synthesis inhibitor with four thiazole and one thiazoline ring is a signature secondary metabolite of *Streptomyces* (5). Derivatives of pristinamycin (for example, RP59500) are currently in advanced clinical testing for combating vancomycin-resistant Gram-positive bacterial infections (6). The heterocyclic rings are likely to arise by cyclization of

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